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FUNGAL METABOLITE OF NAFTAZONE INHIBITS NITRITE PRODUCTION BY ACTIVATED MURINE MACROPHAGES

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Abstract. The fungus *Mucor plumbeus* catalyzes the enzymatic cyclization of Naftazone to naphtho-(1,2-e)-(1,2,4)-triazine-(3H)-one. This compound inhibits the induction and the activity of NO synthase by activated murine peritoneal macrophages.

Naftazone **1** [1,2-naphthoquinone-2-semicarbazone (Etioven^{R})] is an orally active drug which protects the vascular system (1). It inhibits the activity of the constitutive forms of nitric oxide synthases in rat endothelial cells (1). Nitric oxide synthases catalyze the formation of nitric oxide, an efficient endothelium-derivated relaxing factor (2). Nitric oxide is also involved in other physiological processes including neurotransmission (3), cytotoxicity and cell-mediated defense (4). Pharmacokinetic studies on Naftazone have shown that this compound is metabolized in the rat and in man mainly by reduction and glycoconjugation reactions (1). A glucuronide derivative has been identified in the blood and urine of Naftazone-treated rats, and was obtained by incubating Naftazone with rat and human liver microsomes *in vitro* (1). This report describes the isolation of a fungal metabolite of naphtazone and its effects on the induction and activity of the inducible form of nitric oxide synthase in activated murine peritoneal macrophages.

A panel of fungal strains were screened and a strain of *Mucor plumbeus* which converted Naftazone into naphtho-(1,2-*e*)-(1,2,4)-triazine-(*3H*)-one **2** (20% Yiels, 65% conversion) was identified (5). The structure of the metabolite was determined by ^{1}H-and ^{13}C-NMR, and by Mass Spectrometry (5).

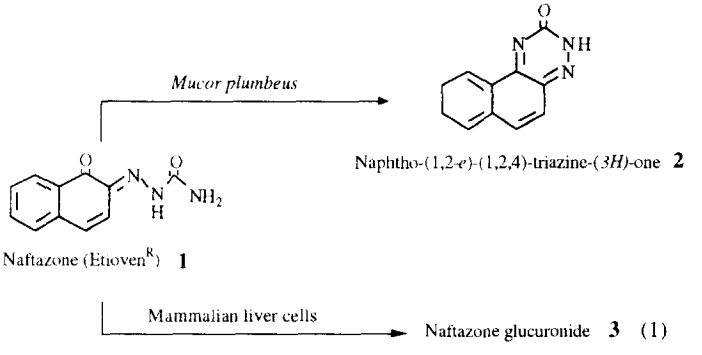

Figure 1: Major metabolites of Naftazone produced by microbial and mammalian metabolism (1).

HPLC analysis shows that compound **2** is not obtained in the fraction containing mainly the glucuronide derivative **3** isolated from the urine of Naftazone-treated rats (1).

The mechanism by which **2** might be formed from **1** was investigated by attempting to dehydrate Naftazone **1**. Under acidic conditions (reflux with toluene in the presence of paratoluene sulfonic acid), no reaction occured. However, refluxing **1** with 3N KOH for 10 hours gave product **2** in 75% yield (9)(figure 2). The mechanism involved would be comparable to the cyclization of 3-ureidoisobutyrate **4** to dihydrothymine **5** by dihydropyrimidase.

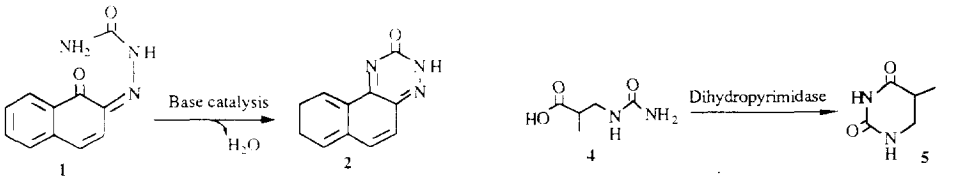

Figure 2: Cyclization of Naftazone (**1**) and 3-ureidoisobutyrate (**4**)

The effect of Naftazone on constitutive endothelial NO synthase (1), prompted us to evaluate its activity and that of compound **2** on the inducible form of murine macrophage NO synthase. In a first type of experiments, each compound was added to macrophage cultures together with LPS, and removed before the addition of NO synthase substrate, arginine (6). Naftazone **1** (10 µM-1 mM) had no effect on the induction of NO synthase (results not shown). In contrast, the triazine derivative **2** inhibited the induction of NO synthase as shown by the decrease of nitrite production by LPS activated-macrophages in a concentration dependent manner (IC_{50} = 50 µM). This inhibition was complete at 100 µM of **2**, and the compound had no toxic effect on the cells as shown by cell viability measured at 540 nm (figure 3).

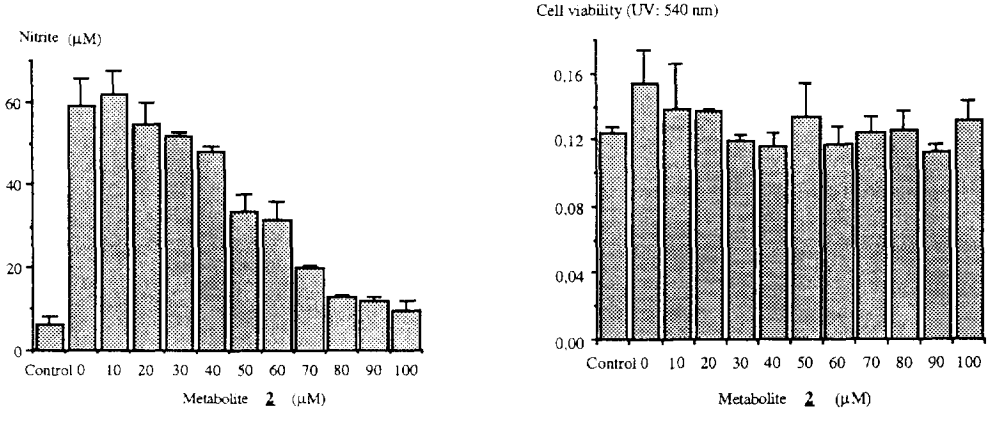

figure 3: Effect of **2** on macrophage NO synthase induction and cell viability. Thioglycollate-elicited peritoneal macrophages from C3H/HeN mice were incubated with 100 ng/ml of LPS and various concentrations of compound **2** for 18 hours at 37°C. The macrophages were washed and incubated in fresh medium containing 2mM L-arginine. Nitrite production and cell viability were measured 24 hours later (7). Each nitrite concentration is the mean of triplicate determinations + standard deviation. Similar results were obtained in three different experiments.

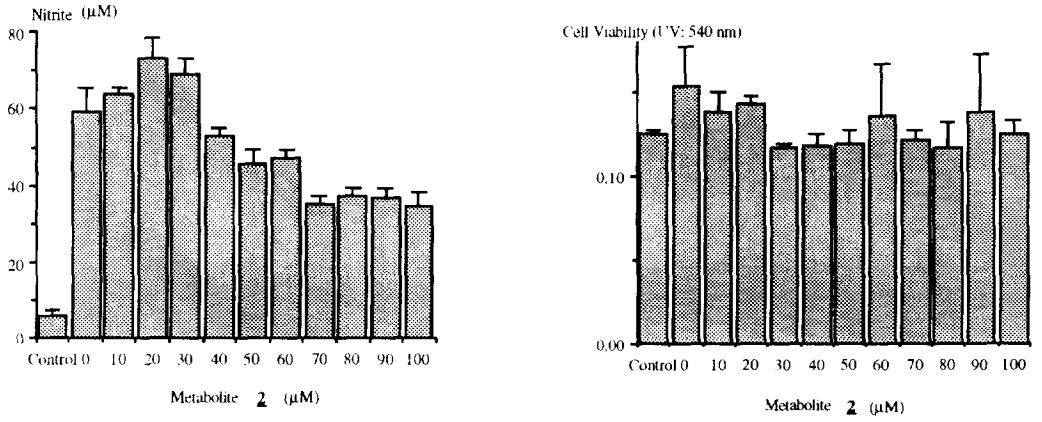

Figure 4: Effect of **2** on the activity of Mφ NO synthase and cell viability. Thioglycollate-elicited peritoneal macrophages from C3H/HeN mice were incubated with 100 ng/ml LPS for 18 hours. Activated-macrophages were washed and placed in fresh medium containing 2mM L-arginine and various concentrations of the microbial derivative **2**. Nitrite production and cell viability were measured 24 hours later (7). Each nitrite concentration is the mean of triplicate determinations ± standard deviation. Similar results were obtained in two different experiments.

A second type of experiments was carried out to evaluate the impact of **2** on the activity of LPS-induced NO synthase. In these experiments, compound **2** is added with arginine at the end of the induction period (6). Above 70 µM of **2**, NO synthase activity is inhibited by 35%, while cell viability is nearly the same between 10 and 100 µM of **2** (figure 4). The decrease in nitrite concentration is not due to a reaction between the formed nitric oxide and compound **2**. HPLC and TLC analysis of a mixture of **2** with gaseous nitric oxide at physiological pH, confirmed the absence of any reaction products (8). In these experiments, Naftazone did not affect nitrite production (results not shown).

Thus, **2** inhibits both the induction and the activity of NO synthase in activated murine peritoneal macrophages. The mechanism of inhibition would be related to an interference with tetrahydrobiopterin **6,** the cofactor of NO synthases (Figure 5). Thus, 7-nitroindazole **8** inhibits both the constitutive bovine brain NO synthase (IC_{50} = 2,5 µM) and the inducible NO synthase from murine macrophages (IC_{50} = 20 µM). This inhibition is competitive with H4-biopterin **6** in both cases (11). Similar results have been reported for 1-phenylimidazole **9** in murine macrophages (IC_{50} = 8 µM) (12). Pyrimidine **7** inhibits the synthesis of H4-biopterin **6** (10,13). As the structures of compounds **2** and **6** have some homologies, the triazine **2** may act by inhibiting the biopterin pathway or the binding of biopterin to NO synthase. This would account for both the inhibition of the activity of a constitutive NO synthase (1) and the inhibition of the induction and the activity of the inducible form.

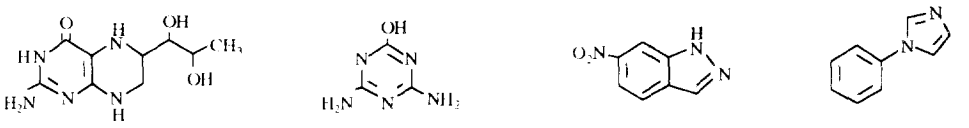

Figure 5: Examples of inhibitors of tetrahydrobiopterin synthesis and tetrahydrobiopterin binding to NO synthase

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References and notes

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5.*Mucor plumbeus* LCM was selected in our laboratory as a high activity hydroxylating fungus. The microorganism was maintained on agar slants (Diagnostic Pasteur, Paris). Spores of *Mucor plumbeus* were used to inoculate the liquid medium containing 1g KH_2PO_4, 0.5g K_2HPO_4, 10g corn steep liquor, 0.5g $MgSO_4$, 2g $NaNO_3$, 0.5g KCl, 0.02g $FeSO_4$ and 30g glucose per liter of distilled water. Cultures were grown in erlenmeyer flasks at 27°C on a rotary shaker (200 rpm). Naftazone **1** (200 mg/l) was added to a three-days old culture of *Mucor plumbeus* and maintained at 27°C and 200 rpm for 15 days, by which time the reaction rate had reached the threshold value of 65%. The biomass was removed by filtration and the filtrate extracted with chloroform; the organic layer was then dried with $MgSO_4$ and evaporated under reduced pressure. The residue was purified by flash column chromatography (silica gel, ethyl acetate / heptane 7:3) to give pure **2** (40mg, 20%). 300-MHz: ^{1}H-NMR (DMSO) 7.45 (1H, d, J=10Hz); 7.65 (1H, d, J=10Hz); 7.75 (1H, m), 7.9 (2H, m); 8.35 (1H, s); 8.8 (1H, d, J= 8Hz). ^{13}C-NMR (DMSO) 79.3, 123.9, 126.2, 128.1, 128.8, 129.5, 129.8, 133.7, 135.9, 136, 154.9. L-SIMS (Matrix: mNBA), 198 $(M+H)^+$; L-SIMS (Matrix: mNBA+LiCl, 20eV), M/z : 407 $(2M+2Li-H)^+$, 401 $(2M+Li)^+$, 210 $(M+2Li-H)^+$, 204 $(M+Li)^+$, 198 $(M+H)^+$; CIMS : 395 $(2M+H)^+$, 198 $(M+H)^+$, EIMS : 187 $(M^{+\cdot})$, 169 $(M^{+\cdot}$- C=O), 141 $(M^{+\cdot}$ - N_2C=O), 127 $(M^{+\cdot}$- N_3C=O). Anal. Calc. for $C_{11}H_7ON_3$ C, 67; H, 3.6; N,21.3; O, 8.1 found. C, 66.9; H, 4.4; N, 19.6; O, 9.16.

6. Male and female C3H/HeN mice (6-8 weeks old, Charles River) were injected intraperitonially with 2 ml thioglycollate three days before harvesting peritoneal cells then the macrophages were cultured. Macrophage monolayers were incubated with LPS for 18 hours, then washed and covered with fresh medium containing L-arginine (2mM). Samples were collected 24 hours after for nitrites and cell viability measurement.

7. Nitrite concentrations were determinated using a procedure based on the Griess reaction and recently modified by: Archer, S.; *FASEB.J.* **1993**, *7*, 349. Cell viability was determined by the method reported by Finter, N. B.; *J.Gen.Virol.* **1969**, 5, 419.

8. Gaseous nitric oxide (N 20, Air liquide , France) was flushed in a solution of **2** (1mM in culture medium) for 2 min. The mixture was analyzed either by TLC (R_f = 0.7 with ethyl acetate on Kieselgel 60F plates, Merck, Germany) or by HPLC (R_t = 6.8, acetonitrile/water/TFA on 5µm hypersil ODS, Shandon, France).

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